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(54) Title: PREVENTION OF LEAKAGE DURING THERMOTROPIC PHASE TRANSITION IN LIPOSOMES AND BIOLOGICAL CELLS

(57) Abstract

Leakage from liposomes or biological cells which occurs upon cooling through the thermotropic phase transition temperature is reduced or eliminated by incorporating thermal hysteresis proteins in the liposome or cell structure. Preferred thermal hysteresis proteins are antifreeze proteins and antifreeze glycoproteins from polar fish species, and chromatographic fraction no. 8 of antifreeze glycoproteins has been found to be particularly effective.

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PREVENTION OF LEAKAGE DURING THERMOTROPIC PHASE TRANSITION IN LIPOSOMES AND BIOLOGICAL CELLS

This invention lies in the field of lipids and the phase transitions of certain lipids from the liquid crystalline phase to the gel phase. In particular, this invention addresses the problem of leakage of internal substances through the membranes of biological cells and liposomes as these bodies pass the phase transition temperature.

GOVERNMENT RIGHTS

This invention was made at least in part with United States Government support under Grant Nos. IBN 93-08581 and DCB89-18822, awarded by the National Science Foundation, and Grant No. N00014-94-1-0379, awarded by the Office of Naval Research. The United States Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Various types of biological substances undergo thermotropic phase transitions between a gel phase and a liquid crystalline phase upon cooling down to temperatures close to but not at or below the freezing temperature. Included among such substances are plant and animal cells, bacteria and liposomes. Cooling of these materials to this phase transition region is a practical and useful means of preservation for purposes such as storage and shipping, since freezing is destructive of the cell structure and dehydration is impractical in many circumstances.

As they enter and pass through this phase transition, plant cells and liposomes have been observed to undergo leakage of their contents. In cells, this leakage destroys viability and texture, while in liposomes there is a loss in usefulness, particularly when the substance which has leaked out is a functional

substance residing in the liposome interior. Liposomes are vesicles formed of concentrically ordered phospholipid bilayers encapsulating an aqueous phase. By incorporating functional molecules such as pharmaceuticals, imaging agents, skin care agents and other useful substances as solutes in the encapsulated aqueous phase, researchers have developed liposomes as useful carriers of these substances. Liposome formulations are thus of interest for such industries as the cosmetics industry and the pharmaceuticals industry, and the leakage problem is a potential obstacle to their stability during storage, shipping and handling.

SUMMARY OF THE INVENTION

It has now been discovered that leakage in both liposomes and biological cells, including both plant cells and animal cells, upon cooling through the thermotropic phase transition temperature is markedly reduced and in some cases eliminated entirely by the treatment of the liposomes or cells with proteins known as antifreeze proteins and antifreeze glycoproteins. Treatment is readily achieved in a variety of ways, including suspending the liposomes or cells in a liquid solution in which the proteins are dissolved. The whole proteins have been found to be more effective than their component parts, including individual amino acids prominently occurring in the protein structure, poly(amino acid) fragments of the proteins, and glycosylated amino acids included in the protein structure, as well as other naturally occurring proteins and common cryogenic preservation agents. It has also been discovered that certain fractions of antifreeze glycoproteins are surprisingly superior to other fractions. This invention thus finds utility in the preservation of animal tissue and of fruits and vegetables and other useful plants, both as food sources

and as sources of extracts for pharmaceutical purposes, cosmetic purposes and other therapeutic and generally beneficial purposes, and also in the preservation of liposomes encapsulating beneficial functional substances.

These and other features and advantages of the invention will become apparent from the description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a plot showing the leakage of a marker compound from liposomes as temperature is lowered through the thermotropic transition temperature. Liposomes treated in accordance with the invention are compared with untreated liposomes.

FIG. 2 is a plot similar to that of FIG. 1, but additionally showing the effect of varying the amount of treatment agent used in the treatment of the liposomes.

FIG. 3 is a plot similar to that of FIG. 1, but additionally showing the effect of using a heat-denatured treatment agent in comparison to one which had not been denatured.

FIG. 4 shows two calorimetric scans, one taken of liposomes treated in accordance with the invention and the other of untreated liposomes.

FIG. 5 is a plot showing the leakage of a marker compound from a different type of liposomes, through both a warming past the thermotropic transition temperature and a cooling back to the initial low temperature.

FIG. 6 is a plot showing the leakage of the marker compound from the same liposomes as FIGS. 1 through 4 as the temperature is raised, rather than lowered, through the thermotropic transition temperature.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

The existence of naturally-occurring macromolecular species known as "antifreeze proteins," "thermal hysteresis proteins," "antifreeze glycoproteins," and "antifreeze polypeptides" is well known and widely reported in the literature. The discovery of antifreeze glycoproteins, for example, was first reported by DeVries, A.L., *et al.*, in "Freezing Resistance in Some Antarctic Fishes," *Science* **163**:1073-1075 (7 March 1969). DeVries, *et al.* observed that various species of fish surviving in water at temperatures averaging -1.87°C over the course of a year did so despite having insufficient levels of sodium chloride and other low molecular weight substances in their blood to depress the freezing point by conventional freezing point depression. DeVries, *et al.* were able to attribute the survival of these species to the presence of certain glycosylated proteins having molecular weights ranging from about 2,500 to about 34,000, which are now referred to as antifreeze glycoproteins or "AFGPs." Further investigations revealed that many species of north temperate and Arctic fishes carry antifreeze compounds in their blood. Some of these compounds are glycoproteins, while others contain no sugar moieties and are referred to as antifreeze polypeptides or proteins ("AFPs"), with molecular weights ranging from about 3,300 to about 12,000. Furthermore, while the compounds lower the freezing point, the melting point remains unaffected, hence the term "thermal hysteresis proteins."

Antifreeze proteins and glycoproteins have been isolated from a wide variety of sources, and these sources and the structures of the various proteins obtained from them have been reported extensively in the literature. The sources include both fish species and non-fish species, and are listed in Tables I and II below.

TABLE I
THERMAL HYSTERESIS PROTEINS OF FISH SPECIES

Protein Type, Composition and Size	Source Fish Species	Trivial Name of Fish species
Antifreeze glycoproteins (AFGPs): contain alanine, threonine and Gal-GalNAc disaccharide: M.W.: 2,600-33,700	Antarctic notothenioids:	
	Pagothenia borchgrevinki	
	Trematomus borchgrevinki	Antarctic cod
	Trematomus bernachii	
	Dissostichus mawsoni	
	Northern ocean gadoids:	
	Gadus agac	Greenland cod
	Gadus morhua	Atlantic cod
	Microgadus tomcod	Atlantic tomcod
	Boreogadus saida	Arctic polar cod
	Eligenus gracilis	Saffron cod
Antifreeze Polypeptides (AFPs), Type I: alanine-rich; M.W.: 3,300-6,000	Righteye flounders:	
	Pseudopleuronectus americanus	Winter flounder
	Limanda ferruginea	Yellowtail flounder
	Cottids:	
	Myoxycephalus scorpius	Shorthorn sculpin
	Myoxycephalus aenaeus	Grubby sculpin
	Myoxycephalus scorpiodes	Arctin sculpin
Antifreeze Polypeptides (AFPs), Type II: cysteine-rich; homologous to C-type lectins; M.W.: 14,000-16,000	Cottid:	
	Hemitripterus americanus	Sea raven
	Osmerus mordex	Smelt
	Clupea harengus harengus	Herring
Antifreeze Polypeptides (AFPs), Type III: no cysteines, and not rich in alanines; M.W.: 5,000-6,700	Eel pouts:	
	Macrozoarces americanus	Ocean pout
	Rhigophila dearborni	Antarctic eel pout
	Lycodes polaris	Arctic eel pout

TABLE II
NON-FISH SOURCES OF
THERMAL HYSTERESIS PROTEINS

A. Insects Other Than Beetles:

Order	Species
Collembola	7 spp.
Plecoptera	*Arcynopteryx compacta*
Orthoptera	*Parcoblata pennsylvanica*
Hemiptera	*Oncopeltus fasciatus*
Mecoptera	*Boreus westwoodi*
Lepidoptera	*Choristoneura fumiferana*

B. Coleoptera (Beetles):

Family	Species
Tenebrionidae	*Tenebrio molitor* *Meracantha contracta* *Uloma impressa* *Platydema sp.*
Elateridae	*Ampedus lineatus* *Ampedus sp.* *Lepidotus discoideus* *Melanotus sp.*
Cucujidae	*Cucujus clavipes*
Pyrochridae	*Dendroides canadensis*
Lampyridae	*Photinus sp.*
Coccinellidae	*Coccinella novemnotata*
Scolytidae	*Ips acuminatus*
Cerambycidae	*Rhagium inquisitor*

C. Non-Insect Arthropods:

Animal	Species
Spiders	*Philodromus sp.* *Clubiona sp.* *Bolyphantes index*
Centipede	*Lithobius forficatus*
Mite	*Alaskozetes antarcticus*

D. Other Invertebrates:

Mussel	*Mytilus edulis*

The proteins which have been the most extensively studied, and which are the preferred proteins for use in the practice of the present invention, are those isolated from fish species. As indicated in Table I, these proteins include both glycosylated proteins (AFGPs) and non-glycosylated proteins (AFPs), and the latter fall within three general categories, designated Type I, Type II, and Type III.

The AFGPs generally consist of a series of repeats of the tripeptide unit alanyl-threonyl-alanyl, with the disaccharide β-D-galactosyl-(1→3)-α-N-acetyl-D-galactosamine attached to the hydroxyl group of the threonine residue, although variations exist. For example, AFGPs of relatively low molecular weight contain proline and arginine residues in place of some of the alanine and threonine residues, respectively. Chromatographic studies of the AFGPs from representative fish species have revealed eight major molecular weight fractions, as indicated in Table III.

TABLE III
Molecular Weight Fractions of AFGPs
From *Pagothenia borchgrevinki*

Fraction No.	Molecular Weight
1	33,700
2	28,800
3	21,500
4	17,000
5	10,500
6	7,900
7	3,500
8	2,600

Preferred AFGPs for purposes of the present invention are those of Fraction No. 8.

The AFPs differ from one another to a larger degree than do the AFGPs. As indicated in Table I, the three types of AFPs differ from each other in their residue content. Type I AFPs are rich in alanine residues (about 65%), with most of the remainder consisting of polar residues such as aspartic acid, glutamic acid, lysine, serine and threonine. The molecular weight ranges from about 3,300 to about 6,000. Type II AFPs are considered to be rich in cysteine (actually half-cysteine) residues, and are homologous to C-type lectins. Type II AFPs from the sea raven contain 7.6% cysteine, 14.4% alanine, 19% total of aspartic and glutamic acids, and 8% threonine. The molecular weight ranges from about 14,000 to about 16,000. Type III AFPs are devoid of cysteine residues and not rich in alanine residues. No conspicuous dominance of any particular amino acid is evident, and the amino acid content is evenly divided between polar and non-polar residues. The molecular weight ranges from about 5,000 to about 6,700. All percents referred to in this paragraph are on a mole basis.

Antifreeze proteins from insects are primarily AFPs of Type II, and typical compositions in terms of amino acid residues are those of the *Choristoneura fumiferana* (spruce budworm) and *Tenebrio molitor* (beetle). These are listed in Table IV, which also includes the amino acid composition of the sea raven for comparison.

TABLE IV
Comparative Amino Acid Compositions of Type II AFPs

Amino Acid Residue	Spruce Budworm Fraction II	Beetle	Sea Raven
Asx	9.5	5.3	10.7
Thr	6.0	2.3	7.9
Ser	13.0	11.1	8.2
Pro	5.0	0.0	6.7
Glx	11.0	12.4	9.1
Gly	15.0	11.4	8.1
Ala	8.0	5.0	14.4
½-Cys	6.0	28.0	7.6
Val	3.0	2.3	1.2
Met	0.0	0.0	5.4
Ile	1.2	1.0	1.7
Leu	6.5	2.2	6.2
Tyr	1.0	0.0	1.2
Phe	2.2	0.0	2.0
Lys	3.1	15.4	2.1
His	0.0	3.1	2.5
Trp	0.0	0.0	2.8
Arg	8.0	0.0	2.3

Antifreeze proteins and glycoproteins can be extracted from the sera or other bodily fluids of fish or insects by conventional means. Isolation and purification of the proteins is readily achievable by

chromatographic means, as well as by absorption, precipitation, and evaporation. Other methods, many of which are described in the literature, will be readily apparent to those skilled in the art.

Thermal hysteresis proteins may also be produced synthetically, either by conventional chemical synthesis methods or by methods involving recombinant DNA. The DNA coding sequences of the genes which form these proteins have been elucidated and are extensively reported. See, for example, DeVries, A.L., *et al.*, *J. Biol. Chem.* **246**:305 (1971); Lin, Y., *et al.*, *Biochem. Biophys. Res. Commun.* **46**:87 (1972); Yang, D.S.C., *et al.*, *Nature* **333**:232 (1988); Lin, Y., *Proc. Natl. Acad. Sci. U.S.A.* **78**:2825 (1981); Davies, P.L., *et al.*, *J. Biol. Chem.* **79**:335 (1982); Gourlie, B., *et al.*, *J. Biol. Chem.* **259**:14960 (1984); Scott, G.K., *et al.*, *Can. J. Fish. Aquat. Sci.* **43**:1028 (1986); Scott, G.K., *et al.*, *J. Mol. Evol.* **27**:29 (1988). Successful microinjection of the AFP gene into species other than its native species has also been reported. See, for example, Zhu, Z., *et al.*, *Angew. Ichthyol.* **1**:31 (1985); Chourrout, D., *et al.*, *Aquaculture* **51**:143 (1986); Dumman, R.A., *et al.*, *Trans. Am. Fish. Soc.* **116**:87 (1987); Fletcher, G.L., *et al.*, *Can. J. Fish Aquat. Sci.* **45**:352 (1988); Maclean, N.D., *et al.*, *Bio Technology* **5**:257 (1987); Stuart, G.W., *et al.*, *Development* **103**:403 (1988); McEvoy, T., *et al.*, *Aquaculture* **68**:27 (1988); Ozato, K, *et al.*, *Cell Differ.* **19**:237 (1986).

As indicated above, one of the fields of application of the present invention is the use of antifreeze proteins and glycoproteins in the treatment of liposomes. Liposomes are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The choice of a particular lipid is generally based on such factors as the desired size and stability of the

resulting liposomes in the bloodstream or other intended mode of administration.

A commonly used lipid component in the liposomes is phosphatidylcholine. Phosphatidylcholines to which a variety of acyl chain groups of varying chain length and degree of saturation have been bonded are commercially available or may be isolated or synthesized by well-known techniques. The more common phosphatidylcholines are those containing saturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22}, although phosphatidylcholines formed from mono- or diunsaturated fatty acids and from mixtures of saturated and unsaturated fatty acids are of use as well. This invention also extends to liposomes formed from phosphonolipids in which the fatty acids are linked to glycerol via ether linkages rather than ester linkages; liposomes formed from sphingomyelin or phospholipids with head groups other than choline, such as ethanolamine, serine, glycerol and inositol; and liposomes formed from cholesterol, diglycerides, ceramides, phosphatidylethanolamine-polyoxyethylene conjugates and phosphatidic acid-polyoxyethylene conjugates. When a sterol such as cholesterol is present, the mole ratio of sterol to phospholipid is generally from about 0.1 to 1.0. Examples of liposome compositions are distearoyl-phosphatidylcholine/cholesterol, dipalmitoylphosphatidyl-choline/cholesterol, and sphingomyelin/cholesterol.

Liposomes may be prepared by a variety of methods described in the literature. Descriptions appear for example in Szoka, *et al.*, *Ann. Rev. Biophys. Bioeng.* 9:467 (1980); U.S. Patent Nos. 4,235,871, 4,501,728, and 4,837,028; the text *Liposomes*, Marc J. Ostro, ed., Marcel Dekker, Inc., New York, 1983, Chapter 1; and Hope, *et al.*, *Chem. Phys. Lip.* 40:89 (1986), all of which are incorporated herein by reference. One method involves dissolving the vesicle-forming lipids in a suitable organic solvent or solvent system and drying the solution

under vacuum or an inert gas to form a thin lipid film. The liposomes produced by this method are multilamellar vesicles which are heterogeneous in size. To achieve a more homogeneous lipid mixture, the film can be redissolved in a suitable solvent such as t-butanol, then lyophilized, covered with an aqueous buffered solution and allowed to hydrate.

Liposomes can be sized by a variety of known techniques. One method is sonication, specifically bath or probe sonication, resulting in a progressive size reduction. Another method is homogenization by the use of shearing energy to fragment large liposomes into smaller ones. A third method is the extrusion of liposomes through a small-pore polycarbonate membrane or an asymmetric ceramic membrane.

Functional compounds such as drugs, cosmetics, imaging agents, and the wide variety of other materials supplying biological utility of some kind can be incorporated into the liposome interior by conventional means. The most common such means are encapsulation and transmembrane potential loading.

Encapsulation of a drug or other functional agent can be achieved by dissolving the agent and the liposome components in an organic solvent in which all species are miscible, then concentrating the resulting solution and evaporating the solvent to a dry film. A buffer is then added to the film and liposomes are formed with the agent incorporated into the vesicle walls. Alternatively, the agent can be dissolved in a buffer and added to a dry film formed solely from the lipid components. The buffer can be any biologically compatible buffer solution. Examples are isotonic saline, phosphate buffered saline, and other low ionic strength buffers. The buffer method will result in liposomes with the agent encapsulated in the aqueous interior of the liposome. In either method, the agent will constitute from about 0.01 ng/mL to about 50 mg/mL of the liposome suspension. The liposomes with

the agent incorporated in the aqueous interior or in the membrane are then optionally sized as described above.

Transmembrane potential loading has been described in detail in U.S. Patent No. 4,885,172, U.S. Patent No. 5,059,421, and U.S. Patent No. 5,171,578, the contents of which are incorporated herein by reference. The method can be used to load any conventional drug which can exist in a charged state when dissolved in an appropriate aqueous medium. The potential is established across the bilayers of the liposomes by producing liposomes having different internal and external media such that a concentration gradient of one or more charged species (such as Na^+, K^+ and/or H^+) is imposed across the bilayers. To load a drug which in ionized form is negatively charged, for example, a liposome created with an inside potential which is positive relative to the outside potential is used.

Biological cells to which this invention is applicable include a wide range of living cells that undergo thermotropic phase transitions. This includes both animal cells and plant cells. Among animal cells, mammalian cells are of particular interest, as well as mammalian tissues, organs and organisms. Examples of mammalian cells to which the invention is applicable are mammalian oocytes, hepatocytes, erythrocytes and leukocytes. Examples of tissues and organs are tissue of livers, hearts, and kidneys, and the organs themselves. Examples of organisms are embryos, and self-sustaining whole animals.

Plant cells to which the present invention is applicable include cells from a wide variety of plants. The cells which will benefit from the invention are those which undergo a thermotropic phase transition in temperature regions above the freezing point. The phenomenon observed in these plant materials is cold shock, or loss through the membrane of low molecular weight constituents. These plant materials include

fruits, vegetables, grains, and other food-source plants, and the type and form of cells which exhibit this behavior range from seeds to germinated seedlings to mature plants, including portions of plants such as leaves, fruits, vegetables, stalks and roots.

The cells, tissues or liposomes can be treated with the antifreeze proteins and glycoproteins in accordance with this invention in a variety of ways. A convenient method is the incubation of the cells or liposomes as a suspension in an aqueous solution of the treatment agent. For suspensions in which the cells or liposomes constitute from about 0.1 mg/mL to about 1 mg/mL of the suspension, the antifreeze proteins or glycoproteins will be present in an amount preferably ranging from about 0.3 mg/mL to about 30 mg/mL of the suspension, more preferably from about 1 mg/mL to about 20 mg/mL, and most preferably from about 3 mg/mL to about 10 mg/mL. The incubation will be performed at a temperature above the phase transition temperature, and the cells or liposomes can be maintained in the suspension until ready for use or concentrated or recovered from the suspension, provided that they are maintained in an environment which will prevent outward diffusion of the antifreeze proteins or glycoproteins. Other means of contacting the cells or liposomes with antifreeze proteins or glycoproteins will be readily apparent to those skilled in the handling of cells, tissues or liposomes.

The following examples are offered by way of illustration rather than limitation.

EXAMPLE 1

This example illustrates the effect of antifreeze glycoproteins and antifreeze proteins in inhibiting leakage from dielaidoylphosphatidylcholine liposomes during a phase transition.

Liposomes were prepared from dielaidoyl-phosphatidylcholine (DEPC) vesicles in a conventional manner, except that carboxyfluorescein was included in the forming solution at a concentration of 200 mM and accordingly trapped inside the resulting liposomes as a marker. Once formed, liposomes were sized by extrusion through polycarbonate filters, using the commercial apparatus produced by Avestin, Inc., Ottawa, Ontario, Canada. Excess carboxyfluorescein not trapped by the liposomes was removed by passing the liposomes through a Sephadex column. The resulting liposome suspensions had a liposome concentration of 20 mg/mL.

Antifreeze glycoproteins obtained from *Trematomus borchgrevinki*, including combined chromatographic fractions 1-8 as well as subcombinations including fractions 2-6, 5-7 and 3-4 were used. In additional experiments, antifreeze proteins (Type I) obtained from *Pseudopleuronectus americanus* were used. The AFPs and AFGPs were tested against a control of untreated liposomes, and comparisons were also made against other potential treatment agents. These included alanine, galactose, N-acetyl galactosamine, glycerol, proline, Rock Fish blood serum and ovotransferrin. Alanine, galactose and N-acetyl galactosamine were included because they are prominent components of AFPGs.

For each experiment in which the liposomes were treated with antifreeze proteins, antifreeze glycoproteins, or any of the comparative substances, the treatment agent was added to the aqueous liposome suspension to achieve a range of final concentrations as shown below. To achieve the phase transition, the liposomes, both treated and control, were placed in a fluorometer and cooled from 20°C to 0°C in a temperature controlled cuvette at a rate of 0.5°C/minute. Leakage was assayed by the increase in fluorescence observed continuously as carboxyfluorescein leaked into the external medium.

The results are listed in Table V below, where the experiments are arranged in increasing order of percent leakage.

TABLE V

Percent Leakage of Carboxyfluorescein From Dielaidoylphosphatidylcholine Vesicles During Cooling Through Phase Transition With Various Treatment Agents

Treatment Agent	Concentration	Percent Leakage
AFGP 1-8*	4 mg/mL	3
AFGP 1-8	1 mg/mL	5
AFGP 2-6	1 mg/mL	9
AFGP 5-7	1 mg/mL	10
AFGP 8	4 mg/mL	21
AFP Type I	1 mg/mL	23
AFGP 3-4	1 mg/mL	33
AFGP 8	1 mg/mL	35
none (control)		55
glycerol	0.4 M	58
N-acetyl galactosamine	1 mg/mL	59
proline	1 mg/mL	62
Rock Fish blood serum	2 mg/mL	70
ovotransferrin	1 mg/mL	81

* For AFGP entries, the number following "AFGP" indicates the AFGP fraction(s).

These tests show that the intact AFGPs have a significant effect in reducing leakage associated with the phase transition, and are consistently more effective than both the AFGP components and fractions and the control. Some of the treatment agents are in fact shown

to increase the leakage rather than reduce it. Leakage curves are shown in FIG. 1, where the open squares (□) represent 1 mg/mL AFGP 1-8, the open triangles (Δ) represent 1 mg/mL AFP (Type I), and the open circles (o) represent the control. The progression in each curve is from right to left as the temperature is lowered through the phase transition temperature of approximately 5°C.

EXAMPLE 2

This example presents further test results on carboxyfluorescein-marked DEPC liposomes, with emphasis on the differences between AFGP fractions, constituent subunits of AFGPs and denatured AFGPs.

The procedures of Example 1 were followed, with bovine serum albumin (BSA) and fractions 6, 7 and 8 of the AFGPs denatured at 80°C for 30 minutes as additional comparative treatment agents. The results are listed in Table VI below.

TABLE VI

Percent Leakage of Carboxyfluorescein From Dielaidoylphosphatidylcholine Vesicles During Cooling Through Phase Transition With Various Treatment Agents

Treatment Agent	Concentration	Percent Leakage
AFGP 8*	10 mg/mL	0
AFGP 8	1 mg/mL	15
AFGP 2-6	1 mg/mL	5
AFGP 5-7	2 mg/mL	9
AFGP 1-5	1 mg/mL	20
AFGP 6	1 mg/mL	40
alanine	1 mg/mL	42
galactose	1 mg/mL	45
BSA	1 mg/mL	47
none (control)		50
denatured AFGP 6-8	1 mg/mL	55
N-acetyl galactosamine	1 mg/mL	59
proline	1 mg/mL	62
Rock Fish blood serum	2 mg/mL	70

* For AFGP entries, the number following "AFGP" indicates the AFGP fraction(s).

These data show that at high concentrations, Fraction 8 leads to complete inhibition of leakage. At a much lower concentration, Fractions 2 through 6 inhibit leakage by as much as 95%. In addition, these data confirm the findings of Example 1, that the components of the AFGPs (alanine, galactose and N-acetyl galactosamine) are not effective, and that heat denaturing destroys the effectiveness of the protein.

The effect of increasing the concentration of AFGP Fraction 8 is shown in FIG. 2. Once again, the

progression in each curve is from right to left as the temperature is lowered through the phase transition temperature of approximately 22°C. The circles (●) represent the control with no treatment agent used; squares (■) represent the sample treated with 1 mg/mL AFGP 8; inverted triangles (▼) represent the sample treated with 2 mg/mL; and triangles (▲) represent the sample treated with 10 mg/mL.

The effect of heat denaturation is shown in FIG. 3. The AFGP fractions used in the data shown in this Figure are Fractions 2-4 combined. The circles represent these fractions used after heat denaturation at 80°C for thirty minutes, while the squares represent the same fractions used without heat denaturation.

FIG. 4 is a calorimetric scan of the liposomes whose test data appears in Table II and in FIGS. 2 and 3. Two scans are shown, the upper scan performed on liposomes treated with AFGP fractions 2-6 from *Dissostichus mawsoni* and the lower scan on liposomes not treated with any treatment agent. The peak seen at approximately 12°C is a melting endotherm which occurs as the hydrocarbon chains melt. The fact that the peak is present in both scans and occurs at the same location leads to the conclusion that the AFGPs do not achieve their leakage inhibition effect by any effect on the phase transition of the liposomes.

EXAMPLE 3

This example illustrates the leakage occurring during a liposome phase transition in the opposite direction, *i.e.*, with an increase in temperature, and the lack of effect of antifreeze glycoproteins on the leakage.

DEPC liposomes were again used in this study. These liposomes were prepared at approximately 23°C, which is above their phase transition temperature. The liposomes

were then cooled rapidly through the phase transition. AFGPs Fractions 5-7 from *Trematomus bernachii* were then added at a concentration of 1 mg/mL. The liposomes were incubated at 4°C for one hour, and then rewarmed to approximately 27°C at a rate of 0.5°C/min. The percent leakage was recorded at two-degree intervals by fluorometer. A parallel test was performed on control liposomes which had not been treated with the AFGPs. During the low-temperature incubation, leakage was minimal, but during the slow rewarming, leakage occurred at a rate indistinguishable from the controls. The liposomes were then recooled slowly through the phase transition temperature. The results are shown in FIG. 5, where the filled circles (●) represent the control data taken in the warming direction; the filled squares (■) represent the test data (AFGP-treated vesicles) taken in the warming direction; the open circles (o) represent the control data taken in the cooling direction; and the open squares (□) represent the test data taken in the cooling direction. The plot indicates that the AFGPs inhibit leakage, but they must be added above the phase transition temperature to achieve this effect.

EXAMPLE 4

This example presents a study of dimyristoyl-phosphatidylcholine (DMPC) liposomes, showing the phase transitions occurring during warming, and comparing the results obtained with and without antifreeze proteins present.

Liposomes were prepared from DMPC at about 4°C in a conventional manner, with carboxyfluorescein as a marker, following the procedure described in Example 1. The antifreeze proteins used for treatment were AFGPs Fractions 2-6 from *Dissostichusus mawsoni*, added to the liposomes at a concentration of 1 mg/mL while the liposomes were still at the low temperature. Once formed, the vesicles were warmed to 28°C at a rate of

0.5°C/min while the percent leakage was recorded at one- or two-degree intervals by fluorometer. This was followed by cooling the vesicles back down to 3°C, again at 0.5°C/min while leakage measurements were recorded by fluorometer. The results are shown in FIG. 6, where the circles represent the control liposomes and the squares the AFGP-treated liposomes.

The data in FIG. 6 show that the AFGPs had essentially no effect on leakage during the phase transition in the direction of increasing temperature.

The foregoing is offered primarily for purposes of illustration. It will be readily apparent to those skilled in the art that the choice of proteins, proportions, methods of treatment, and other parameters of the invention described herein may be further modified or substituted in various ways without departing from the spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A method for the treatment of biological materials that undergo a thermotropic phase transition, said biological materials selected from the group consisting of biological cells, biological tissues, and liposomes containing biologically active substances in the interior of said liposomes, to reduce leakage of substances from the interior of said biological materials during thermotropic phase transitions, said method comprising contacting said biological materials with a leakage-reducing amount of one or more thermal hysteresis proteins to a sufficient degree to effect such reduction in leakage.

2. A method in accordance with claim 1 in which said biological materials are members selected from the group consisting of plant cells, plant tissues, and liposomes containing biologically active substances in the interior of said liposomes.

3. A method for the treatment of liposomes containing biologically active substances in the interior of said liposomes to reduce leakage of said biologically active substances during thermotropic phase transitions, said method comprising contacting said liposomes with a leakage-reducing amount of one or more thermal hysteresis proteins to incorporate said thermal hysteresis proteins into said liposomes.

4. A method in accordance with claim 3 in which said one or more thermal hysteresis proteins are proteins having the molecular structure of thermal hysteresis proteins isolated and purified from a polar fish species.

5. A method in accordance with claim 4 in which said polar fish species is a member selected from the group consisting of Antarctic notothenioids, northern ocean gadoids, righteye flounders, cottids and eel pouts.

6. A method in accordance with claim 3 in which said one or more thermal hysteresis proteins are members selected from the group consisting of:

(a) antifreeze glycoproteins isolated and purified from a member selected from the group consisting of Pagothenia borchgrevinki, Trematomus borchgrevinki, Trematomus bernachii, and Dissostichus mawsoni;

(b) Type I antifreeze polypeptides isolated and purified from a member selected from the group consisting of Pseudopleuronectus americanus and Limanda ferruginea;

(c) Type II antifreeze polypeptides isolated and purified from Hemitripterus americanus; and

(d) Type III antifreeze polypeptides isolated and purified from a member selected from the group consisting of Macrozoarces americanus, Rhigophila dearborni and Lycodes polaris.

7. A method in accordance with claim 3 in which said one or more thermal hysteresis proteins are members selected from the group consisting of:

(a) antifreeze glycoproteins isolated and purified from a member selected from the group consisting of Dissostichus mawsoni and Trematomus bernachii;

(b) Type I antifreeze polypeptides isolated and purified from Pseudopleuronectus americanus;

(c) Type II antifreeze polypeptides isolated and purified from Hemitripterus americanus; and

(d) Type III antifreeze polypeptides isolated and purified from Macrozoarces americanus.

8. A method in accordance with claim 3 in which said one or more thermal hysteresis proteins are antifreeze glycoproteins.

9. A method in accordance with claim 3 in which said one or more thermal hysteresis proteins are antifreeze glycoproteins molecular weight fraction 8, as separated by chromatography.

10. A method in accordance with claim 3 in which said liposomes have lipid components that are phosphatidylcholines.

11. A method in accordance with claim 3 in which said liposomes have lipid components selected from the group consisting of dielaidoylphosphatidylcholine and dimyristoylphosphatidylcholine.

12. A method in accordance with claim 3 comprising incubating said liposomes with an aqueous solution of said thermal hysteresis proteins to form an aqueous suspension of said liposomes.

13. A method in accordance with claim 12 in which said thermal hysteresis proteins comprise from about 0.3 mg/mL to about 30 mg/mL of said suspension.

14. A method in accordance with claim 12 in which said thermal hysteresis proteins comprise from about 1 mg/mL to about 20 mg/mL of said suspension.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16520

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 9/127
US CL : 424/450
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450; 514/12, 21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US, A, 5358,931 (RUBINSKY ET AL.) 25 October 1994, abstract, Tables and claims.	1 & 2 -------- 1 - 14
X -- Y	US, A, 5,118,792 (WARREN ET AL.) 02 June 1992, column 2, line 14, columns 3-13 and claims.	1 & 2 -------- 1 - 14
Y	US, A, 5,225,182 (SHARMA) 06 July 1993, column 1, lines 20-28.	1 - 14
Y	US, A, 5,173,219 (KIM) 22 December 1992, column 1, lines 32-36.	1 - 14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 FEBRUARY 1996	Date of mailing of the international search report 21 MAR 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer G. S. KISHORE Telephone No. (703) 308-2351

Form PCT/ISA/210 (second sheet)(July 1992)*

FIG. 1

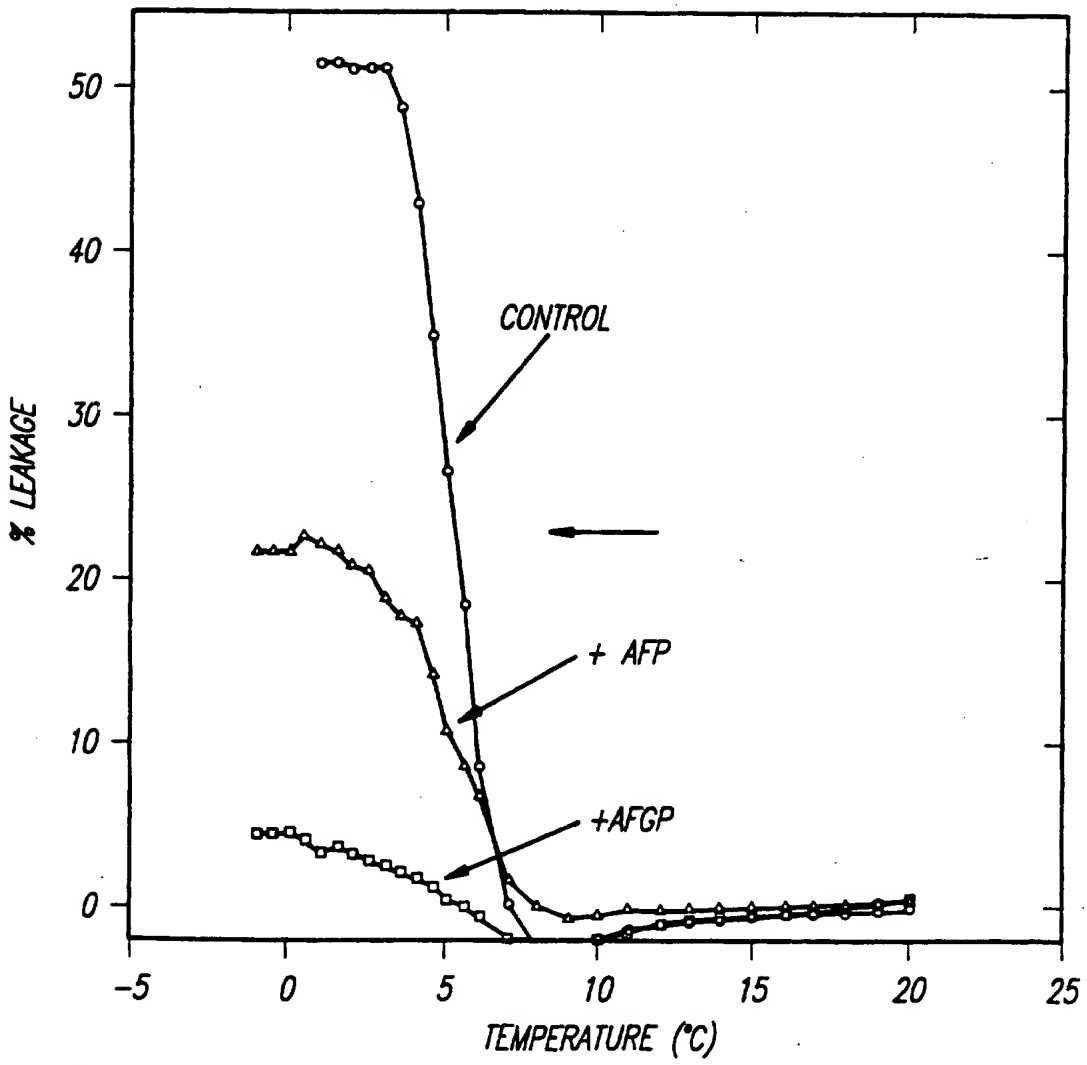

50
40
30
20
10
0
% LEAKAGE
CONTROL
+ AFP
+AFGP
-5
0
5
10
15
20
25
TEMPERATURE (°C)

FIG. 2

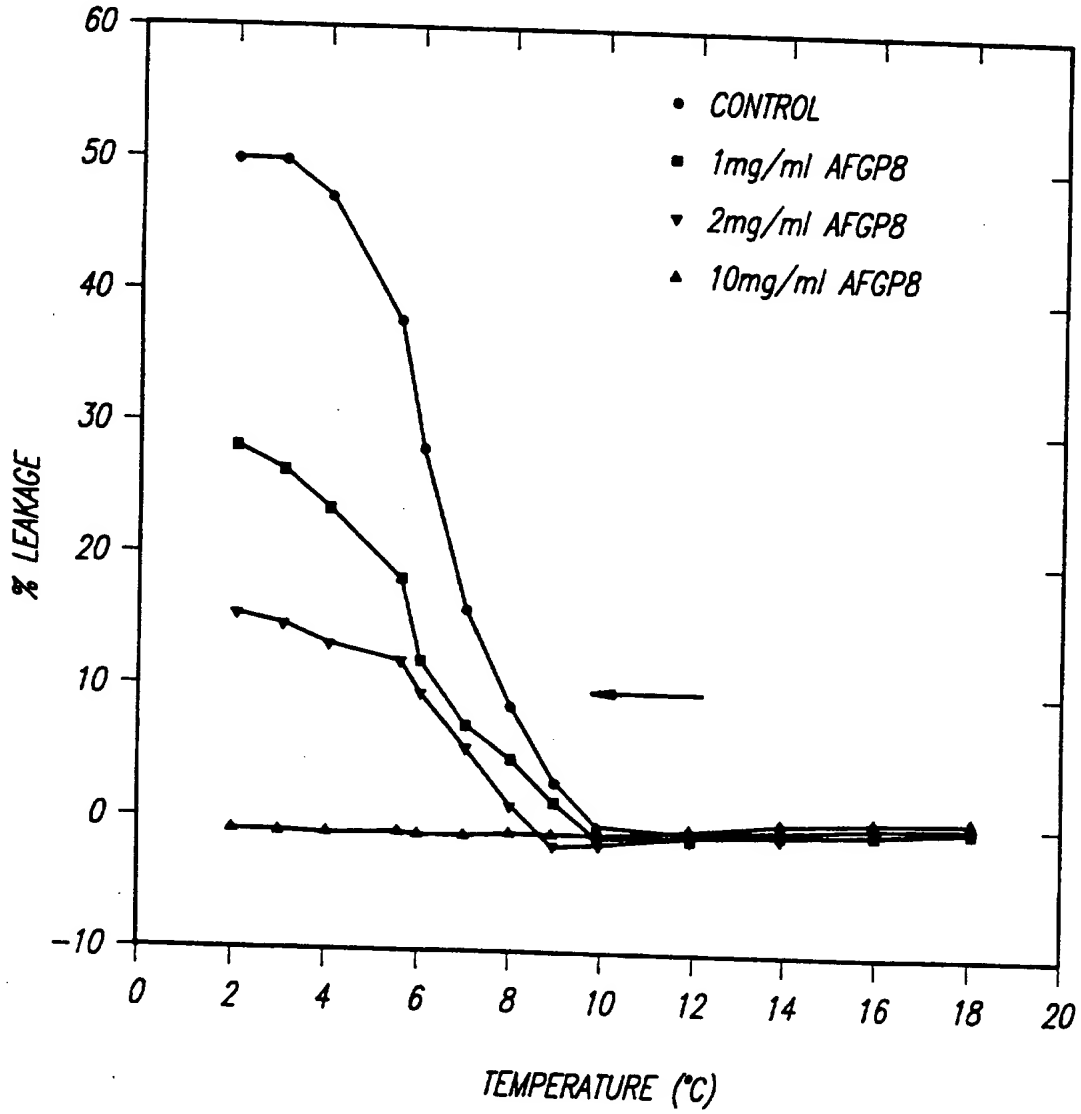

60
50
40
30
20
10
0
-10
% LEAKAGE
0
2
4
6
8
10
12
14
16
18
20
TEMPERATURE (°C)
CONTROL
1mg/ml AFGP8
2mg/ml AFGP8
10mg/ml AFGP8

FIG. 3

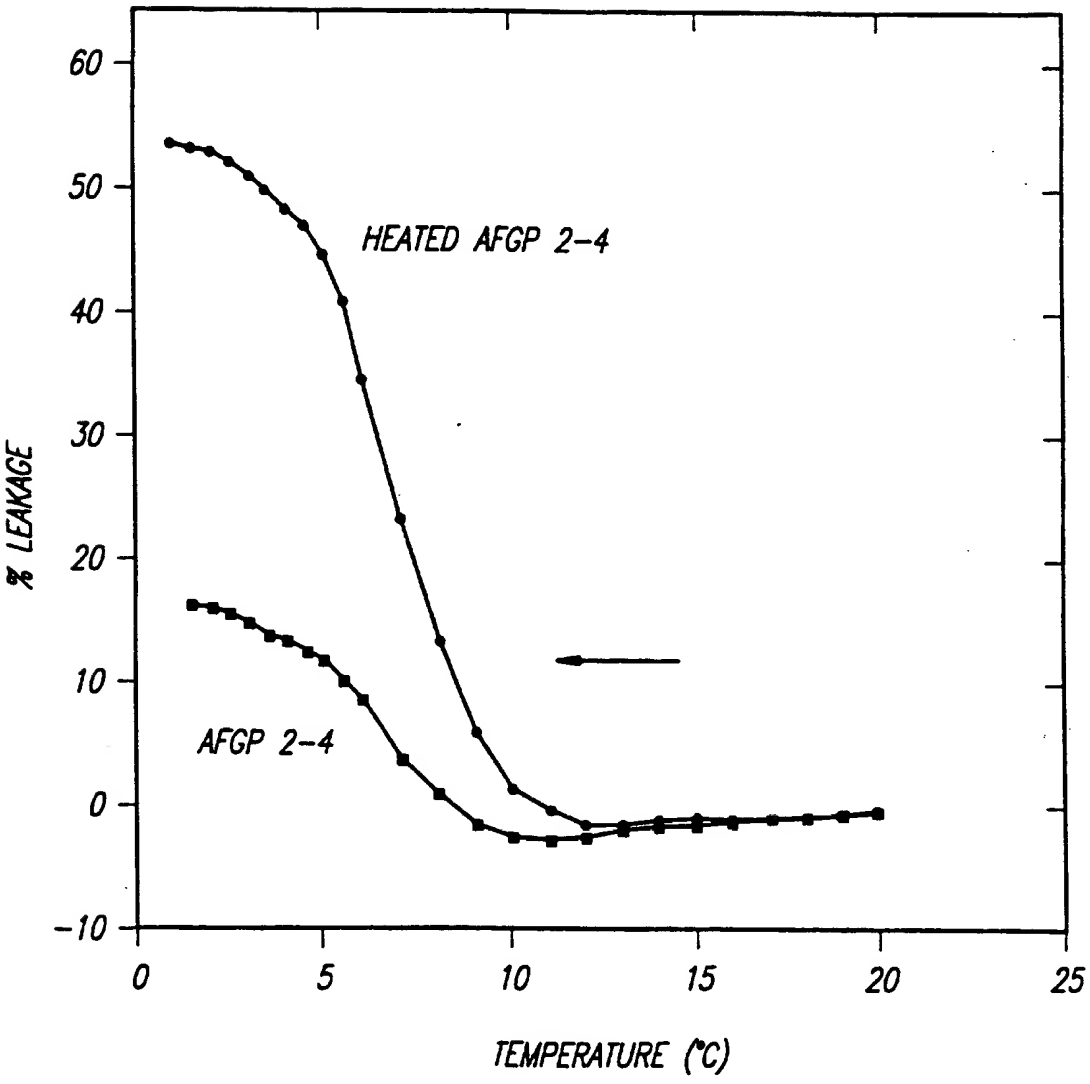

60
50
40
30
20
10
0
-10
% LEAKAGE
HEATED AFGP 2-4
AFGP 2-4
0
5
10
15
20
25
TEMPERATURE (°C)

FIG. 4

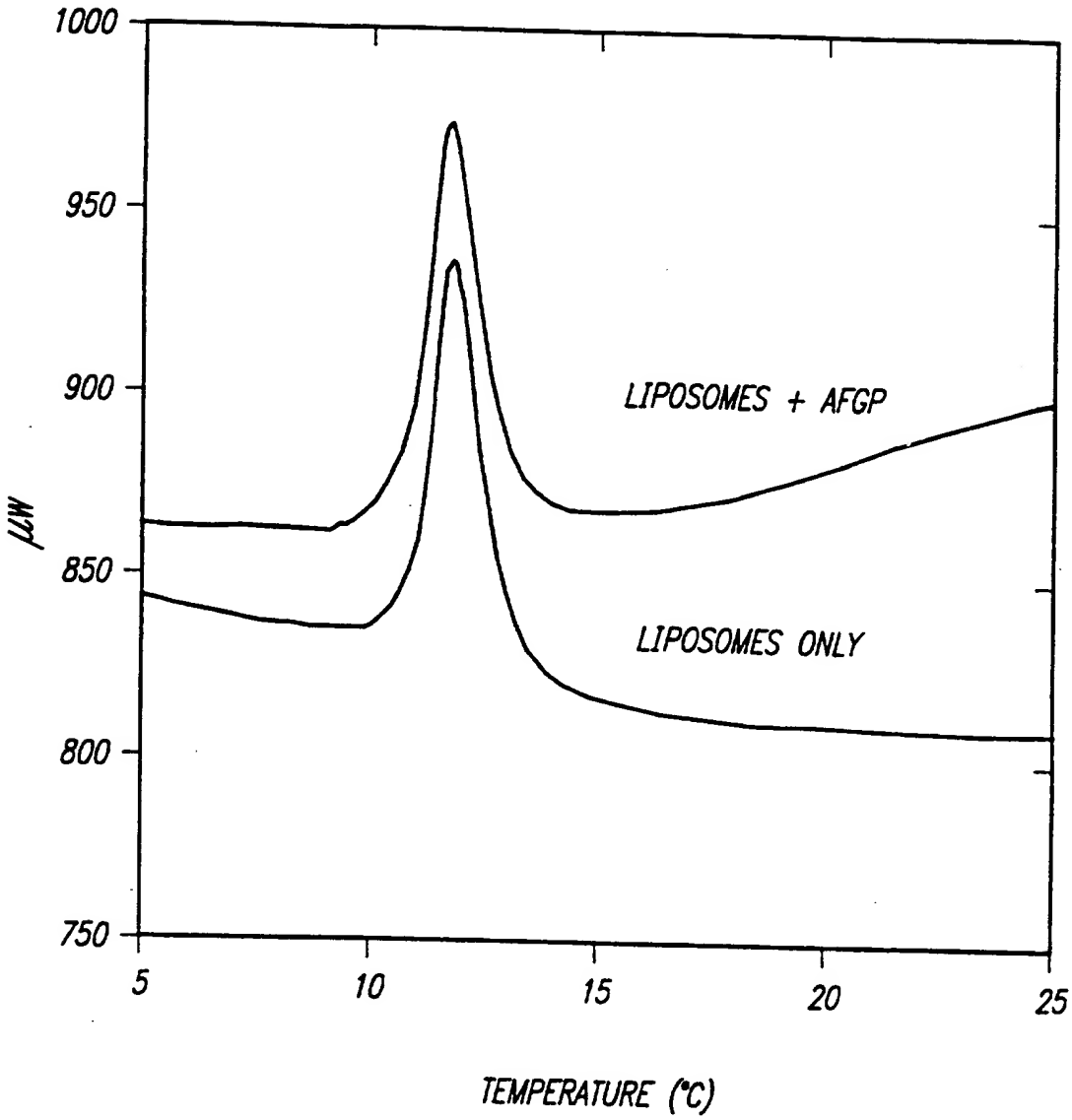

1000
950
900
850
800
750
μW
5
10
15
20
25
TEMPERATURE (°C)
LIPOSOMES + AFGP
LIPOSOMES ONLY

FIG. 5

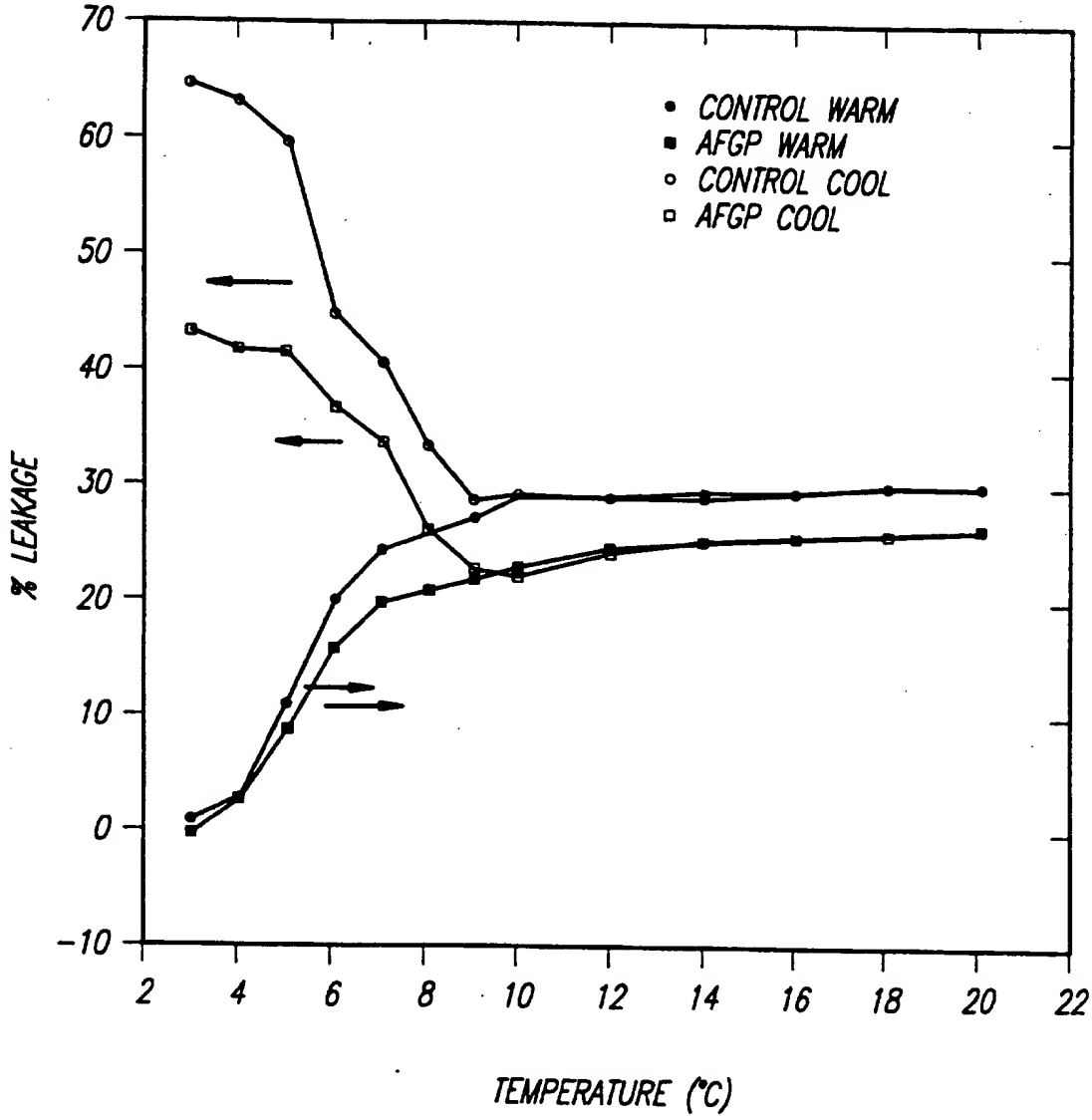
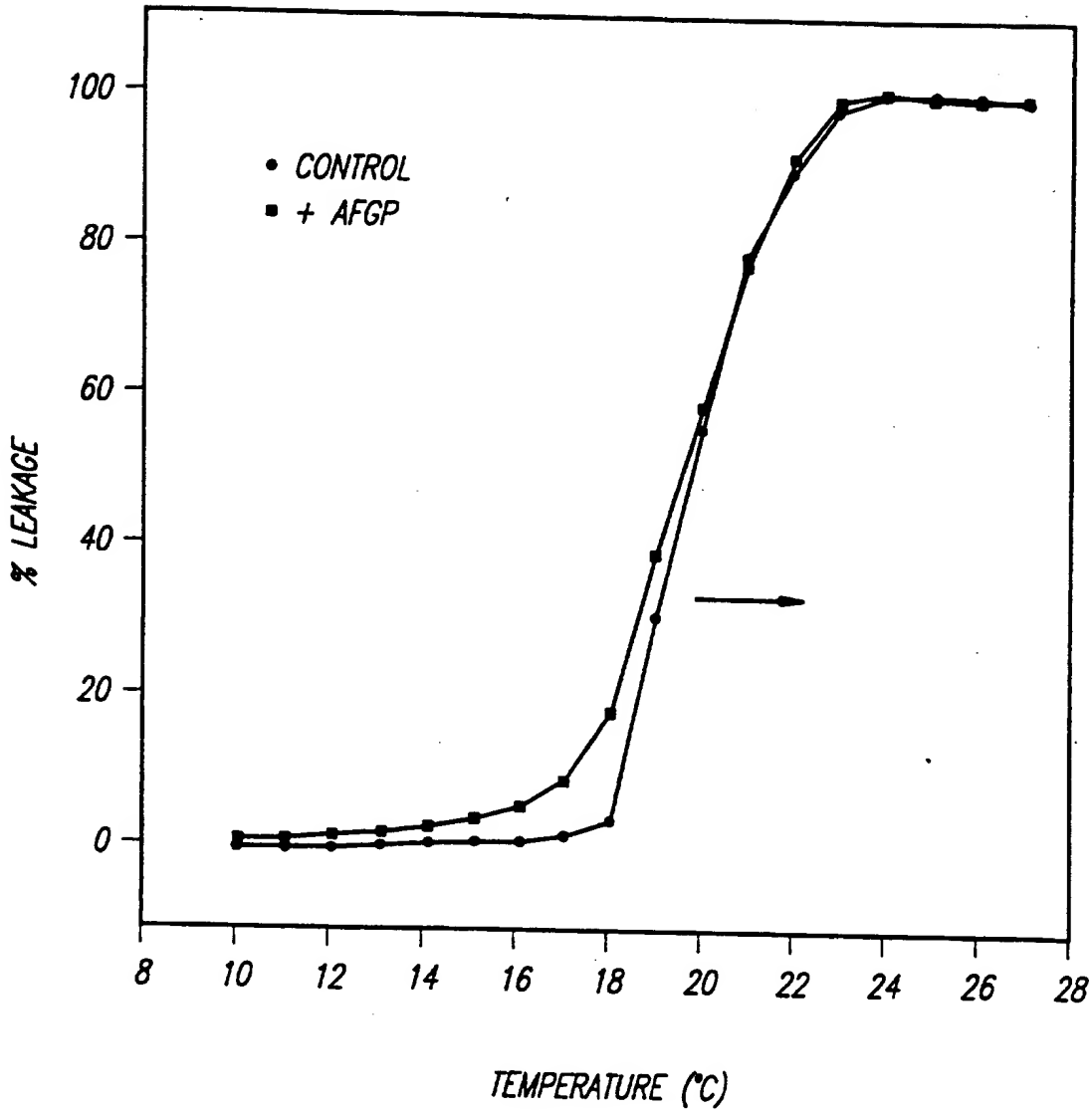

70
60
50
40
30
20
10
0
-10
% LEAKAGE
2
4
6
8
10
12
14
16
18
20
22
TEMPERATURE (°C)
CONTROL WARM
AFGP WARM
CONTROL COOL
AFGP COOL

FIG. 6


100
80
60
40
20
0
% LEAKAGE
• CONTROL
▪ + AFGP
8
10
12
14
16
18
20
22
24
26
28
TEMPERATURE (°C)